Advances in Brief

Platelet: Transporter of Vascular Endothelial Growth Factor

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Abstract

In animal models, growth of tumors and their metastases is dependent on factors that stimulate vessel formation (angiogenesis). Most clinical studies confirm the importance of angiogenesis for cancer growth in patients. Recent studies on circulating angiogenic factors in patients have focused on serum vascular endothelial growth factor (VEGF) levels in a variety of cancer types.

We measured serum VEGF concentrations and blood counts in 27 breast cancer patients during each of 6 cycles of chemotherapy with doxorubicin and cyclophosphamide supported by granulocyte macrophage colony-stimulating factor.

Serum VEGF concentrations highly correlated with platelet counts during chemotherapy ($r = 0.8$; $P < 0.01$). In particular, during the first treatment cycle, after an initial episode of thrombocytopenia, a strong platelet rebound coincided closely with a serum VEGF peak ($r = 0.9$; $P < 0.01$). In addition, plasma VEGF concentrations from 15 other cancer patients and 30 healthy volunteers were 5- to 8-fold lower than their corresponding serum VEGF concentrations ($P < 0.001$). Activation of platelets increased the VEGF content 8–10 times. These findings demonstrate that VEGF is released by platelets during serum preparation.

In this study, we found evidence for VEGF transport by platelets, indicating that serum VEGF concentrations reflect mainly platelet counts rather than tumor burden in cancer patients, as reported earlier. Platelets, known to be important for wound healing, have also been reported to contribute to metastasis formation and tumor growth in animal models. Indeed, tumors can be regarded as never-healing wounds. Our data suggest that platelets may have a stimulating role on angiogenesis-dependent tumor growth through their function as transporters of VEGF.

Introduction

Angiogenesis is required for tumor growth and metastasis (1). Tumor cells release or induce the release of growth factors stimulating proliferation, migration, and tube formation of capillary endothelial cells (2). Microvessel density has been shown to be an independent prognostic factor for survival in stage I and II breast cancer (3, 4). Most, but not all, studies confirm these findings not only in breast cancer, but in a wide variety of cancer types (5, 6).

VEGF,[3] which is a mitogenic, chemotactic permeability factor for endothelial cells (7), is expressed in breast cancer, and its tissue concentration correlates with prognosis (8, 9). Recently, VEGF was found to be released by platelets when stimulated by thrombin *in vitro* (10). Even when megakaryocytes were cultured in serum-free medium, they could release VEGF on stimulation, indicating a constitutive production of VEGF by the megakaryocytes.

Angiogenic growth factors have also been measured in serum and urine of cancer patients. Basic fibroblast growth factor, another angiogenic growth factor, was found to be elevated in the urine of 38% of cancer patients (11). Elevated serum VEGF concentrations have been detected in 13.1% of 175 breast cancer patients (12), with higher concentrations in patients with stage III compared to those with stage I or II disease. In addition, increased serum VEGF concentrations were significantly associated with high microvessel density and VEGF expression of primary tumors in these patients.

In the present study, we measured serum VEGF concentrations in 27 breast cancer patients during 6 consecutive cycles of chemotherapy supported by GM-CSF.

Initially, we hypothesized that serum VEGF concentrations originated from breast cancer cells. We expected circulating VEGF levels to decrease during treatment, because a clinical response rate of almost 100% was achieved in this group of patients (13). In contrast, we found serum VEGF fluctuations that coincided with platelet counts, indicating platelets as major VEGF transporters. Following the recent observation of Mohle *et al.* (10) that activated platelets release VEGF *in vitro*, we investigated whether VEGF release by platelets could be responsible for the serum VEGF concentrations.

Patients and Methods

Patients. We studied 19 patients with locally advanced breast cancer who participated in a Phase II trial and were treated with 4–6 cycles of moderately high doses of doxorubicin (90 mg/m^2) and cyclophosphamide (1000 mg/m^2) supported

[3] The abbreviations used are: VEGF, vascular endothelial growth factor; GM-CSF, granulocyte macrophage colony-stimulating factor.

by GM-CSF (250 μg/m^2/days 2–12; Ref. 14). Subsequently, these patients underwent mastectomy and radiotherapy. Eight patients with metastatic breast cancer received an identical chemotherapy regimen.

Blood and Serum Samples. Venous blood was drawn from the patients on at least a weekly schedule. Blood counts were performed with an automated blood Coulter counter. Immediately after clot formation (20–30 min at room temperature), the tubes were centrifuged at 3000 rpm, and serum was removed and stored at −20°C. Plasma (EDTA blood) and serum from 15 patients with a variety of cancer types and from 30 healthy volunteers were taken according to routine procedures. To find the origin of circulating VEGF, platelet suspensions were stimulated with 6C9, a monoclonal antibody that is known to activate platelets and induces aggregation and release of the contents of the α and dense granules of platelets (15).

VEGF Concentrations. VEGF concentrations were measured with a quantitative sandwich enzyme immunoassay (R&D Systems, Abingdon, United Kingdom). Briefly, serum samples were incubated on microtiter plates precoated with a murine monoclonal antibody against VEGF. The plates were washed, and an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. After a second washing procedure, a substrate solution was added. This substrate colored in proportion to the amount of VEGF, which was bound to the microtiter plate. No significant differences were found when measurements were done in duplicate.

Statistical Analysis. The correlation coefficients of serum VEGF concentrations and platelet or leukocyte counts were calculated by linear regression. The unpaired Student's t test was used to detect differences between VEGF concentrations in serum and plasma. Statistical significance was determined as $P < 0.01$. Variations of the means were given as SE.

Results

Serum VEGF concentrations were measured in the sera of 19 patients with locally advanced breast cancer and 8 patients with metastatic breast cancer during chemotherapy. Pretreatment concentrations were 330 ± 206 pg/ml. At day 8 of the chemotherapy cycle, serum VEGF decreased to 129 ± 85 pg/ml ($P < 0.001$). On days 15–20, an increase in serum VEGF occurred (493 ± 275 pg/ml; $P < 0.001$). We observed these fluctuations in serum VEGF levels during all six cycles of chemotherapy, although the peak was lower during later courses.

A high correlation ($r = 0.8$; $P < 0.01$) between serum VEGF levels and platelet counts was found for the entire patient group. In Fig. 1, the mean values of the serum VEGF concentrations of all patients and their platelet counts are shown during six cycles of chemotherapy. During the first chemotherapy cycle, when the platelet rebound was most obvious, the correlation was even higher ($r = 0.9$; $P < 0.01$). Leukocyte counts did not correlate with serum VEGF concentrations during chemotherapy ($r < 0.3$; data not shown). In addition, no correlation between tumor size and pretreatment VEGF concentrations was found ($r < 0.3$).

Serum VEGF *versus* Plasma VEGF. Comparison of serum and plasma from a group of 15 patients with a variety of cancer types showed that plasma VEGF was significantly lower than serum VEGF ($P < 0.001$; Fig. 2). We compared the VEGF concentrations of plasma with those of serum of 30 healthy blood donors and found that VEGF concentrations were 5- to 8-fold lower in plasma than they were in the serum of the same individuals ($P < 0.001$; Fig. 2). In this group of volunteers, serum VEGF levels also correlated significantly with platelet counts ($r = 0.7$; $P < 0.01$). In addition, the induction of platelet activation *in vitro* by use of the monoclonal antibody 6C9 increased the VEGF content of the platelet suspension significantly by 8 to 10-fold.

Pretreatment serum VEGF concentrations from breast cancer patients did not differ significantly from those of healthy donors (330 *versus* 287 pg/ml; $P = 0.2$). VEGF concentrations in citrate platelet-rich and -poor plasma are equal to those of EDTA plasma, indicating that EDTA has no effect on the VEGF concentrations (data not shown).

Discussion

Although a correlation between circulating angiogenic factors and the angiogenic phenotype of a tumor and its metastatic pattern is to be expected, it has never been proven to exist thus far. In the present study, we found a high correlation between serum VEGF concentrations and platelet counts. In addition, plasma levels were significantly lower than serum VEGF levels, and activation of platelets increased VEGF release, indicating that serum VEGF largely reflects platelet VEGF and may not result from tumor production.

At this point, we need to reconsider the interpretation of VEGF measurements in the sera of cancer patients as reported by others (12, 16–19). Thus far, they were unable to show that higher serum VEGF concentrations correlate significantly with worse prognoses. Platelet counts are not reported in these studies, but it is well known that cancer patients frequently have higher platelet counts and consume more platelets than healthy volunteers (20). In light of our present results, it would be essential to investigate the value of plasma VEGF levels in cancer patients as a potential marker of prognosis.

The effect of platelets on tumor growth and wound healing has been studied extensively. Indeed, platelet activation, which occurs during wound healing, has been shown to result in the release of numerous factors, including platelet-derived endothelial cell growth factor, platelet-derived growth factor, prostaglandins, fibrinogen, thrombospondin, and platelet factor 4 (21). Most of these factors have proangiogenic or antiangiogenic properties (22). It has been reported that the formation and growth of metastases in animals can be inhibited by thrombopenia, antiplatelet therapy, or administration of anticoagulants (23–26). Wound healing and tumor growth are similar processes, both angiogenesis dependent, although tumors do not heal (27). High turnover of platelets and fibrinogen in cancer patients has been reported since the early 1970s (20). Recently, thrombocytosis in lung cancer patients has been shown to be a negative prognostic factor for survival (28).

Together, these data emphasize the importance of platelets in tumor biology. Further research should pay attention to the role of different angiogenesis factors released by platelets and the specific interaction of platelets with tumor endothelium.

Fig. 1 Serum VEGF concentrations and platelet counts during chemotherapy in 27 breast cancer patients (*c*, chemotherapy cycle; *Pre Treatm.*, blood samples drawn before start of chemotherapy; *error bars*, SE.
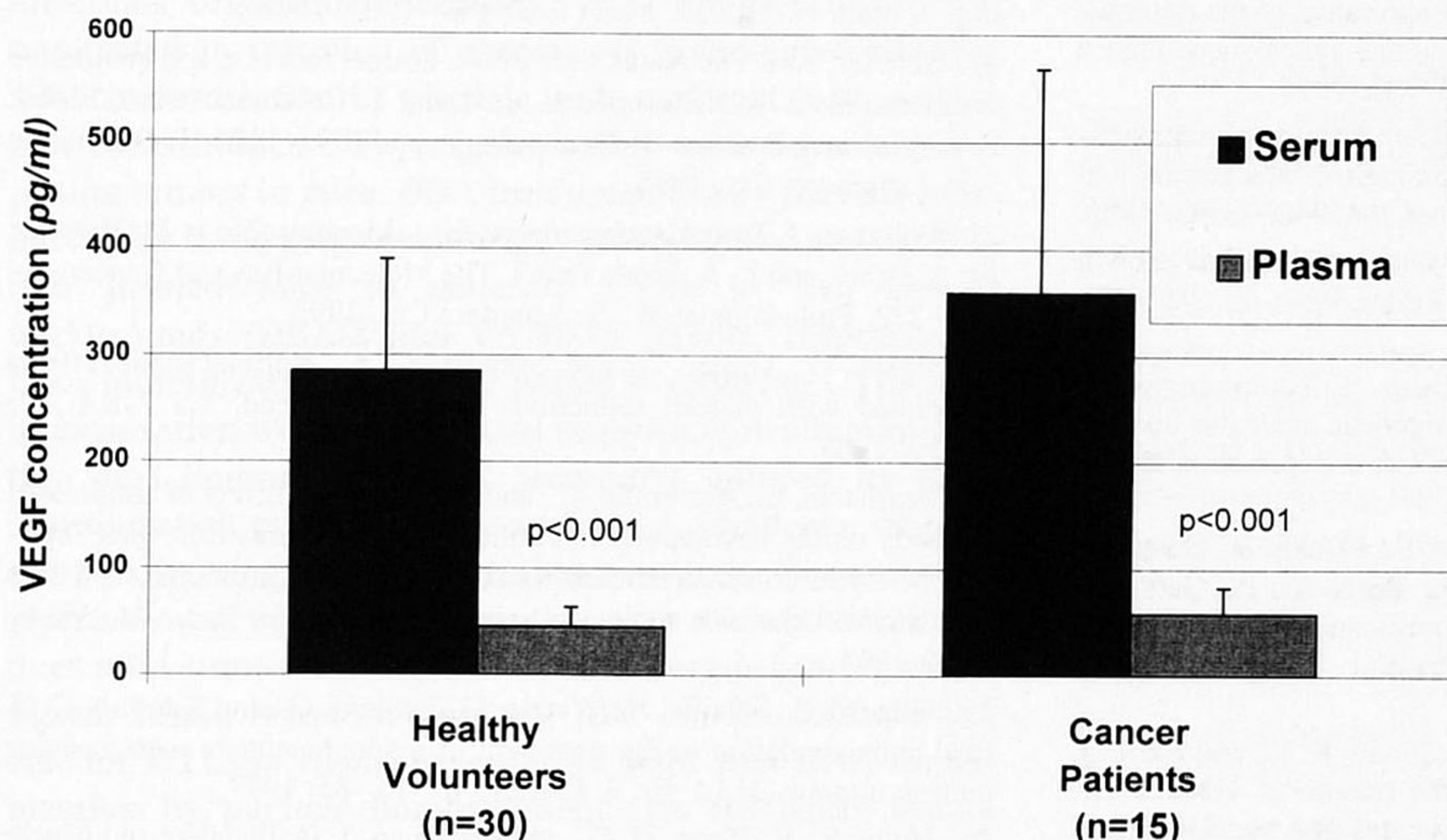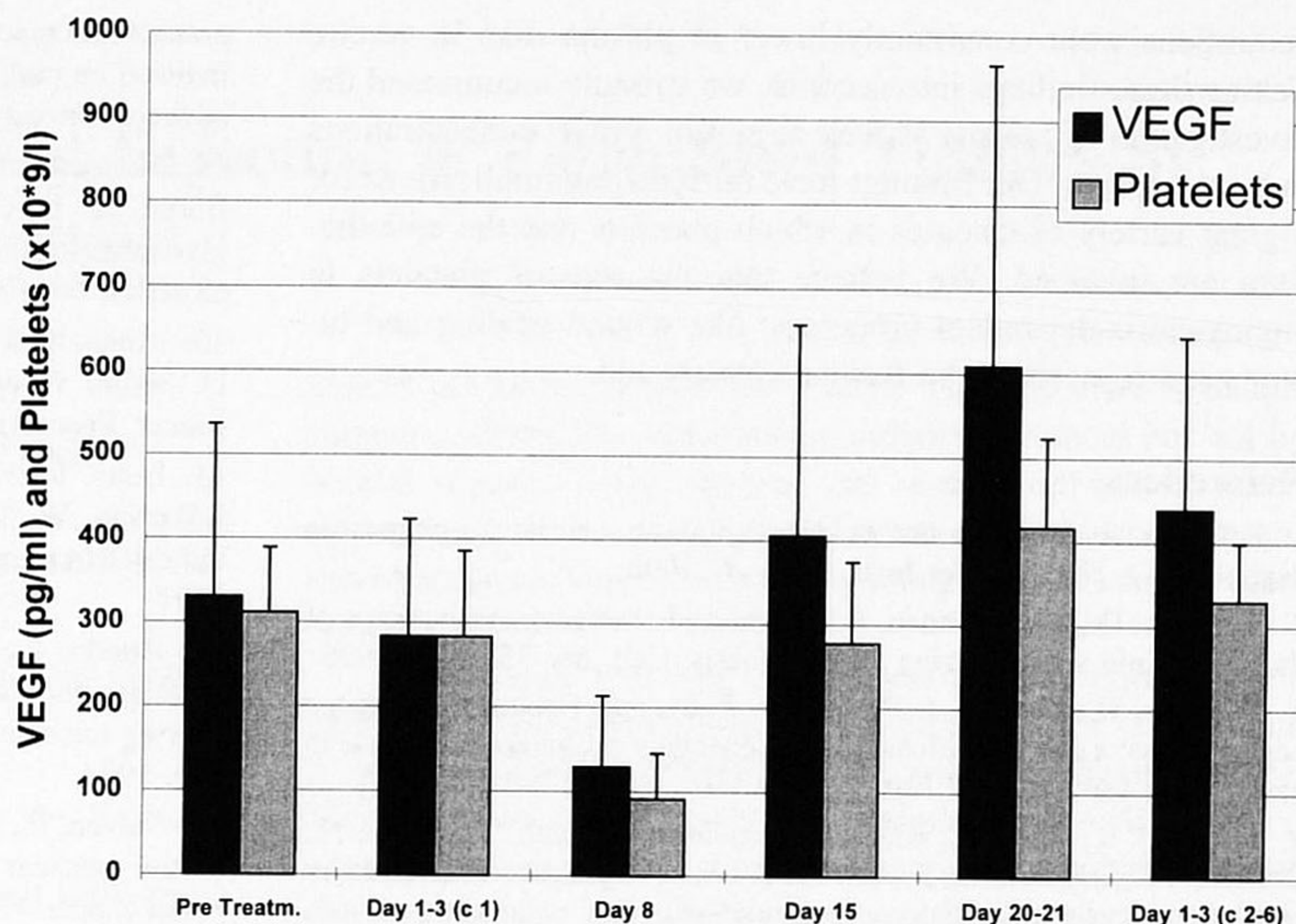

Fig. 2 Serum VEGF compared to plasma VEGF concentrations in patients with a variety of cancer types and healthy volunteers. *Error bars*, SE.

In addition, we must take into account the role of some supportive agents added to chemotherapeutic regimens of cancer patients. For example, GM-CSF is used as a bone marrow-supportive agent, as in the present patient group. It has already been reported that treatment of Lewis lung carcinoma-bearing mice with GM-CSF stimulates metastatic growth (29). It might well be that GM-CSF causes an increase in circulating platelets that are subsequently trapped by both the primary and metastatic tumors. These trapped platelets, in turn, may release angiogenic factors such as VEGF. Earlier, we reported that GM-CSF induced a platelet rebound in the first two cycles of chemotherapy in breast cancer patients (14).

The hemopoietic growth factor thrombopoietin, which stimulates platelet production (30), is currently being studied in clinical cancer trials as well. Thrombopoietin needs to be studied also in view of a potential indirect effect on tumor biology.

Rebound of platelets, which is generally present during the first cycles of myelotoxic chemotherapy, might have an un-

wanted effect on primary tumors. Because administration of chemotherapy causes endothelial damage in the tumors, one may assume that these tumors act like real wounds and trap and activate circulating platelets, supporting regrowth of the tumor by delivery of VEGF during the second half of the cycle. To avoid the undesired effects of VEGF delivered by platelets, especially during their rebound, there might be a place for anti-VEGF treatment or for agents or drugs preventing platelet aggregation and activation during platelet recovery. Indeed, it has been proposed to use thromboprophylactic drugs in the treatment of cancer patients not only to prevent thromboembolism, but also for their unexplained antitumor effects (31). The latter may now be explained by VEGF and other angiogenic factors transported by platelets.

In conclusion, in this study, we show that platelets are the major physiological transporters of VEGF in blood. In breast cancer patients and in healthy volunteers, serum VEGF concentrations highly correlated with platelet counts, and VEGF con-

centrations were consistently lower in plasma than in serum. Taking these findings into account, we strongly recommend the investigation of plasma as well as serum VEGF concentrations in these patients. Our findings have far-reaching implications for a great variety of diseases in which platelets and the endothelium are involved. We believe that the role of platelets in angiogenesis-dependent processes, like wound healing and tumor growth, needs to be further investigated.

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